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Establishment of physical environment in solid state fermentation for the production of glucoamylase using Box-Behnken design

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ABSTRACT

Aims: Glucoamylase is an industrially important enzyme and is second to the proteases in worldwide distribution and sales among industrial enzymes. The aim of this study was to maximize its production from a GRAS strain *Aspergillus oryzae* IIB-6 using cheap medium by solid state fermentation.
Methodology and results: Different carbon and nitrogen sources were evaluated as an additional supplement to wheat bran. The response surface methodology (RSM) was employed for optimization of most important physical parameters viz., pH, temperature and inoculum density as their monitoring is very difficult because of the solid nature of the substrate. Box-Behnken factorial design based on three levels and three variables was employed to obtain optimal combination of three independent variables. Maltose and peptone were found to be the best additional carbon and nitrogen sources. The optimal conditions for pH, temperature and inoculum size were 5.29, 36.2 °C and 17.7% (v/w), respectively as prescribed by second-order polynomial quadratic model. Under optimized conditions, the experimental value of enzyme activity was 7750.02 U/gds, which is very close to 7713.04 U/gds predicted by the model. R^2 was 99.99% which indicates a good agreement between experimental and predicted values.
Conclusion, significance and impact of study: The analysis of variance showed that *p*-values of the coefficients for linear effects and square effects of all the variables were <0.0001, suggesting these variables are highly significant for the enzyme production. Overall 23.01% increase in enzyme production was achieved by RSM optimization which can be proposed for food and pharmaceutical industries.

Keywords: Glucoamylase, *Aspergillus oryzae*, physical parameters, Box-Behnken factorial design, Response Surface Methodology.

INTRODUCTION

Glucoamylase (GA, EC 3.2.1.3) is an industrially important enzyme that release glucose from non-reducing end of starch and related oligosaccharides, involving in hydrolysis of α-1,4-glycosidic linkage faster than α-1,6 and α-1,3 linkages (Gudi *et al*., 2013). GA currently used in food and pharmaceutical industries, in the production of glucose syrup, high fructose corn syrup and alcohol. Although it can be produced from variety of organisms; however, filamentous fungi have a great potential for extracellular production of this enzyme (Zambre, 2010). GA is exclusively produced in industries mainly from *Aspergillus niger* (Slivinski *et al*., 2011; Dharani and Kumaran 2012; Gudi *et al*., 2013), *A. oryzae* (Zambare, 2010; Parbat and Singhal 2011; Sughanthi *et al*., 2011), *A. awamori* (Negi and Banerjee, 2010), *A. flavus* (Koc and Metin, 2010) and *A. niveus* (Maller *et al*., 2011) probably due to their ubiquitous nature and non-fastidious nutritional requirements. Solid state fermentation (SSF) is an old technology and has been used since 2600 BC. However, in recent year SSF has emerged as a well developed biotechnological tool for the production of enzymes (Bhatnagar *et al.,* 2010). The production of amylases in SSF is affected by a variety of physical factors, including the incubation temperature, pH, aeration, particle size, age and size of inoculum. Therefore, after selecting a culture medium for amylase production, the fermentation conditions must be optimized to improve enzyme production at a low production cost (Dharani and Kumaran 2012).

Factorial experiments are commonly used in all research fields such as industrial biology, clinical science, social science, food science, physical and engineering science (Tekindal *et al*., 2012). The experimental design methodology is a very economic way for extracting the maximum amount of complex information, a significant experimental time saving factor and moreover, it saves the material used for analyses and personal costs as well. There is a relationship between the number of variables and the total cost of the experiment. As the number of variables increases, so does the number of design points

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required (Kincl *et al*., 2005). The factor effect is the change in the response variable for certain levels of the factors. In some experiments, due to interaction between factors, the difference in the response between the levels of one factor is not the same for all levels of another factor (Montgomery, 2001). The experiment investigates the effects of various combinations of two or more factors and their levels on the response variable. In factorial experiments, the main effects of the factors and their interaction are assessed (Siklar, 2000). The Box-Behnken experimental design, developed by Box and Behnken (1960), is a useful method for developing second-order response surface models. It is based on the construction of balanced incomplete block designs and requires at least three levels for each factor. In this experimental design, the level of one of the factors is fixed at the center level while combinations of all levels of the other factors are applied (Kocabas, 2001; Myers and Montgomery, 2002). In the present study, Box-Behnken factorial design was employed to investigate the effect of three most important physical parameters i.e., pH, temperature and inoculum size on the production of GA from *A. oryzae* IIB-6 in solid state fermentation. To our knowledge, use of RSM for the optimization of GA production has yet not been reported.

MATERIALS AND METHODS

Chemicals

The analytical grade chemicals used in the present study, were purchased directly from Sigma (USA), E-Merck (Germany), Acros (Belgium), Fluka (Switzerland) and BDH (UK).

Microorganism and culture maintenance

A. oryzae IIB-6 was isolated from soil samples collected from various localities of Doha (Qatar) as described previously (Fatima and Ali, 2012). It was propagated on PDA (4%) slants, stored at 4 °C in cold cabinet (P342; Griffin) and renewed twice a month.

Inoculum preparation

Ten mL of sterilized 0.005% Monoxal-OT (Di-octylester of sodium sulpho succinic acid) were aseptically transferred to a 3-5 days old culture having profuse growth. The clumps of spores were broken with the help of a sterilized inoculating wire loop. The homogeneous suspension was made by gently shaking the tube.

Measurement of viable spore density

Equal volumes (100 µL) of spore suspension and 0.4% trypan blue stain were mixed. For measurement of viable spore density, 10 µL of this mixture was applied to haemocytometre (Marienfeld, Germany) and visualized under the microscope. The density of the spores was measured in accordance to Sharma (1989).

Solid state fermentation

Static cultures were established in 250 mL Erlenmeyer flasks containing wheat bran 7.4 g with 67% moisture level. The flasks were autoclaved (Model: KT-40L, ALP Co, Ltd 3-3-10, Midorigaoka, Hamara-shi Tokyo Japan) for 15 min at 121 °C, 15 lbs/in² pressures. Filter sterilized mineral salts solution containing additional trace elements $FeSO_4 \cdot 7H_2O$ 0.0134; $MgSO_4 \cdot 7H_2O$ 0.67; $(NH_4)_2SO_4$ 2.68; KH_2PO_4 0.402; K_2HPO_4 0.938 (mg/gds) was added at ambient temperature under aseptic conditions. The supplementary organic carbon and nitrogen sources (1%) used were glucose, fructose, maltose, xylose, sucrose, lactose and tryptone, peptone, casein, yeast extract, malt extract, beef extract, respectively. The flasks were inoculated with 10% (v/w) spore suspension and incubated for 86 h.

Estimation of moisture contents

Moisture contents (%) of the substrate were estimated (Kunamneni *et al*., 2005) by drying various amounts of wheat bran to constant weight at 80±2 °C for 15 min in hot air oven (Model: 545804, Memmert 854 Schwabach Germany) and the dry weight was recorded. To fix the initial moisture content of the solid medium, the substrate was soaked with the appropriate quantity of mineral salts solution. The sample was then dried as described above and moisture content was calculated as follows:

$$Moisture\ conents\ (\%) = \frac{(wt.of\ wheat\ bran - dry\ wt.)}{dry\ wt.} \times 100$$

Extraction of enzyme

At the end of fermentation, sodium acetate buffer (0.05 M, pH 5) in a 1:10 ratio was added to individual Erlenmeyer flasks and agitated thoroughly on rotary shaking (200 rpm) incubator (Model: 10×400 XX2.C Sanyo Gallenkamp PLC, UK) for 60 min at 30±2 °C (Ghildyal *et al*., 1991). The contents were filtered through muslin cloth. The filtrate was centrifuged at 4±2 °C, 13,000 rpm (25,900×*g*) for 15 min and the clear supernatant was used for further analysis.

Enzyme assay

GA was assayed according to Caldwell *et al*. (1968) and the amount of reducing sugar liberated was determined by 3, 5-dinitrosalicylic acid method (Miller, 1959) by measuring color intensity at 546 nm on a UV/VIS double beam scanning spectrophotometer (Cecil CE 100-series, Aquarius Inc., London, UK) using D-glucose as standard. One unit of activity was the amount of enzyme that liberates 1 mg of glucose from 5% soluble starch at 60 °C, pH 5.0, per min and enzyme activity was expressed in term of units per gram dry substrate (U/gds). All experiments were carried out in triplicate and the average enzymatic activity was taken by CoStat 3.03 (CoHort software, Berkeley, CA94701).

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Box-Behnken factorial design

To investigate the cumulative effect of physical parameters viz. pH, temperature and inoculum size experiments were carried out according to Box-Behnken factorial design. MINITAB-16 (PA, USA) was used to generate design with three factors and three levels, including three replicates at the centre point, resulting in total 15 treatment with combinations of independent variables pH, temperature and inoculum size. The variables were investigated at three (–1, 0, +1) levels (Table 1) and were coded according to (Canettieri *et al*., 2007). The responses of the input variables were evaluated as a function of GA production coded by Ŷ (U/gds). Predicted GA activity was calculated by a second order polynomial quadratic model:

$$\hat{Y} = \beta_o + \sum_{i=1}^{K} \beta_i x_i + \sum_{i=1}^{K} \beta_{ii} x_i^2 + \sum_{i,j=1} \beta_{ij} x_i x_j + \varepsilon$$

where, Ŷ was the predicted response used as a dependent variable; *xi* (*i* = 1, 2 and 3) were the input predictors; β_o is the regression coefficient at center point; β_i (*i* = 1, 2 and 3) is the linear coefficient; β_{ii} (*i* = 1, 2 and 3) is the quadratic coefficient; β_{ij} (*i* = 1, 2 and 3; *j*=i,.....,3) is the second order interaction coefficient and ε is the experimental error. The Student's *t*-test was employed to check the statistical significance of the regression coefficients. The Fisher's *F*-test for ANOVA was performed on experimental data to evaluate the statistical significance of the model.

Table 1: Range and levels of physical parameters of SSF process.

Parameters	Code	Units	Range and level			
			-1	0	+1	ΔX*
pH	X1	-	4	5.25	6.5	1.25
Temperature	X2	°C	20	37.5	55	17.5
Inoculum size	X3	%	5	17.5	30	12.5

*ΔX is the increment of the experimental factor actual values corresponding to one unit of the coded variable.

RESULTS AND DISCUSSION

Evaluation of additional carbon and nitrogen sources

Wheat bran was supplemented with various organic carbon and nitrogen sources (1% w/w) to study their effect on enzyme production. Supplementation with maltose enhanced the GA production while all the other carbon sources adversely affected the enzyme synthesis (Figure 1A). Similar results were found when maltose (1%) was used in the production of this enzyme from *A. tamari* (Moreira *et al*., 1999). It might be due to the fact that the other sugars used i.e. glucose, fructose, lactose and xylose are monosaccharides hence, act as only carbon sources to the growing culture while maltose is diasaccharide with two glucose units liked via α-1, 4-glycosidic linkage, it acts not only as carbon source but also as an inducer. As the GA is an inductive enzyme that hydrolyzed α-1,4-glycosidic linkage faster than α-1,6 and α-1,3 linkages (Gudi *et al*., 2013) therefore, the addition of maltose enhanced the enzyme production. However, in sucrose, glucose and fructose are linked via α-1, β-2-glycosidic linkage which cannot be hydrolyzed by the enzyme. Al-Ghanimi *et al*. (2007) used 1.5% maltose as an inducer for the production of GA from *A. ustus*. However, Kareem *et al*. (2009) reported the adverse effect of maltose for the GA production from cowpea waste by *A. oryzae*. Lalitha *et al*. (2012) achieved maximum enzyme production on supplementing the corn cob residues with fructose but in the present study fructose was found to be least effective for the GA production. Hence, maltose was selected as best carbon source for the production of enzyme. Among the nitrogen sources (Figure 1B) peptone gave the highest enzyme activity. Yeast extract also increased the enzyme yield but 4.61% less than peptone. Similar increase in the yield of GA from *A. niger* growing in a solid-state fermentation on rice bran supplemented with peptone was reported by Pandey *et al*. (1994). Similarly, Balkan *et al*. (2011) observed less amylase activity with yeast extract as compared to peptone when they supplemented wheat bran with them at 1% concentration. Therefore, peptone was chosen as the best nitrogen source for the subsequent studies.

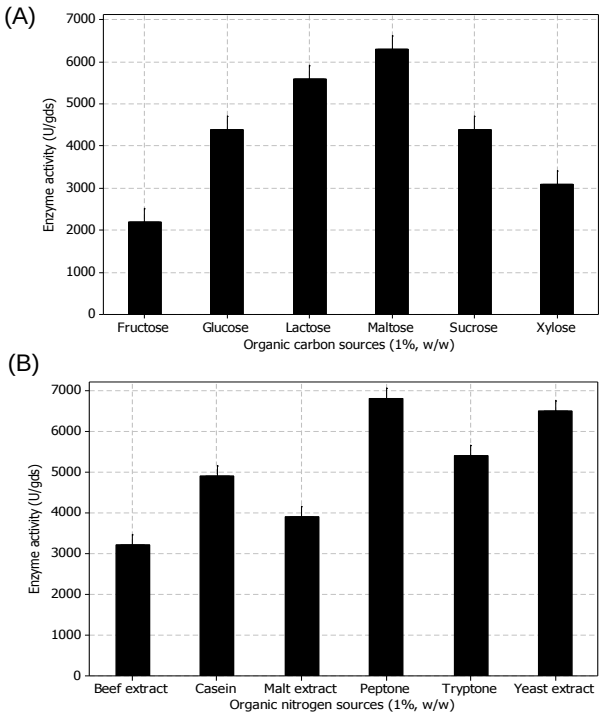

Figure 1: Effect of supplementary organic carbon (A) and nitrogen (B) sources (1% w/w) after 86 h of incubation at 30±2 °C (pH 5.0). Y-error bar shows standard error (S.E) among three parallel replicates (*n*=3).

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Optimization by response surface methodology

Box-Behnken factorial design

Experiments were carried out to optimize the effects of process variables such as pH, temperature and inoculum size for GA production according to Box-Behnken factorial design. For predicting the optimal level mathematically, second order polynomial regression model was fitted to the experimental results of GA production, as:

$$\hat{Y}=-18522.5+7637X1+179.8X2+314.9X3-720.1X1^2-2.5X2^2-8.7X3^2+0.2X1X2-1.2X1X3$$

The regression equation showed that GA production (U/gds) is a function of pH, temperature and inoculum size. Table 2 describes the process conditions and experimentally (Y) measured enzyme activity (U/gds) with predicted (Ŷ) values. Experimental results showed maximum activity of 7712 U/gds while predicted value was 7708 U/gds (run number 10). The residual (e = 3.33) was due to few percentages of the total variations which could not be explained by the model.

Table 2: Box-Behnken factorial design of physical parameters of SSF in actual and coded units for the response of enzyme activity along with it's observed and predicted values.

Run No.	pH (X1)	Temperature °C (X2)	Inoculum size % (X3)	Enzyme activity (U/gds)		Residual e = Y- Ŷ
				Observed response (Y)	Predicted response (Ŷ)	
1	4 (-1)	37.5 (0)	30 (+1)	5200±0.20	5207	-7
2	6.5 (+1)	55 (+1)	17.5 (0)	5777±0.21	5766	11
3	4 (-1)	20 (-1)	17.5 (0)	5858±0.22	5868	-10
4	5.25 (0)	37.5 (0)	17.5 (0)	7708±0.40	7708	0
5	4 (-1)	55 (+1)	17.5 (0)	5623±0.23	5606	17
6	6.5 (+1)	37.5 (0)	5 (-1)	5273±0.19	5266	7
7	5.25 (0)	55 (+1)	30 (+1)	5490±0.15	5499	-9
8	6.5 (+1)	37.5 (0)	30 (+1)	5321±0.18	5322	-1
9	5.25 (0)	55 (+1)	5 (-1)	5376±0.16	5393	-17
10	5.25 (0)	37.5 (0)	17.5 (0)	7712±0.50	7708	4
11	6.5 (+1)	20 (-1)	17.5 (0)	5997±0.20	6013	-16
12	5.25 (0)	37.5 (0)	17.5 (0)	7706±0.49	7708	-2
13	4 (-1)	37.5 (0)	5 (-1)	5077±0.20	5076	1
14	5.25 (0)	20 (-1)	5 (-1)	5669±0.16	5659	10
15	5.25 (0)	20 (-1)	30 (+1)	5758±0.14	5740	18

*± indicates the standard deviation (±S.D) of parallel replicates (n=3). Each mean value differ significantly at p≤0.05%.

Measurement of the effectiveness of the model

A good estimated regression model should explain the variation of the dependent variable (U/gds) in the sample. There are certain tests (Figure 2 A-D) about the model parameters that can help the experimenter in measuring the effectiveness of the model. These tests require for the error term or residuals (e) to be normally and independently distributed with mean zero and variance. The normality of error may be examined by plotting a histogram of residuals (Figure 2C) or a normal probability plot (Figure 2A). Normal probability plots allow a more quantitative assessment of the normality of the distribution of residuals. In this study, the residuals are normally distributed along the straight line as shown in Figure 2 A so, the normality assumption is satisfied. The error term is the difference between observed values (Y) and the corresponding predicted values (Ŷ). As a result of this assumption, observations are also normally and independently distributed. Figure 2 B indicates a constant variance showing that the variance was independent of GA production which again support the assumptions of the model. In this study, the value of correlation co-efficient (R^2) was 0.9999 and residual versus order plot (Figure 2D) look good, the data are randomly scattered around zero with no pattern which indicates a high level of correlation between experimental and predicted values. It was also indicated that 99.99% of the independent variables such as pH, temperature and inoculum size were supported by the dependent variable or response in term of enzyme activity (U/gds). R^2 is also a valid measure for the fit of model and indicated that only 0.1% of the total variations were not explained by the model. The high value (99.96%) of the adjusted R^2 also indicates a high significance of the model (Khuri and Cornell, 1987; Alam *et al*., 2008).

Analysis of variance (ANOVA)

The ANOVA results of response surface quadratic regression model for GA activity (U/gds) are given in Table 3. In general the *F*-value should be higher than *p*-value for the prediction to be significant. In this study,

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among the main, squared and interaction effects of the variables on the GA activity, the main and squared effects have very high *F*-values and low *p*-values (<0.0001) which played a major role for GA production than the interaction effects which were insignificant. The lack-of-fit of the model also found to be insignificant which is strongly desirable for the adequate model.

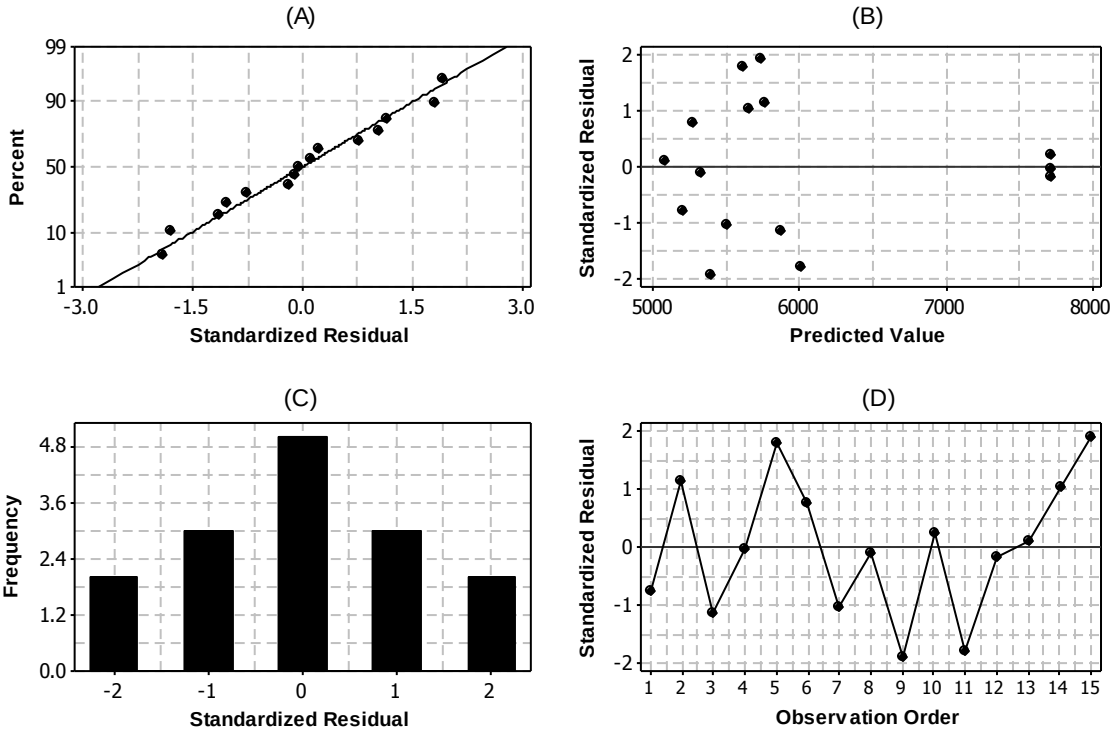

Figure 2: Standardized residual plots for the production of GA (U/gds) from *A. oryzae* IIB-6 in SSF. (A) Normal probability (%) plot. (B) Standardized residual versus predicted values. (C) Standardized residual versus frequency. (D) Standardized residual versus observed order.

Table 3: ANOVA for response surface quadratic regression model of enzyme activity (U/gds).

Source	Degree of freedom	Seq sum of square	Adj sum of square	Adj mean square	*F*-value	*p*-value
Regression	9	1225435	12254350	1361594	4194.25	<0.0001**
Linear	3	193029	5811437	1937146	5967.18	<0.0001**
Square	3	12059702	12059702	4019901	12382.9	<0.0001**
Interaction	3	1619	1619	540	1.66	0.289*
Residual error	5	1623	1623	325		
Lack-of-fit	3	1604	1604	535	57.30	0.17*
Pure error	2	19	19	9		
Total	14	12255073				

R-Sq = 99.99%, R-Sq (pred) = 99.79%, R-Sq (adj) = 99.96%, *Non-significant at $p>0.05$%, **Significant at $p<0.05$%

Main and interactive effects of physical parameters on enzyme production under SSF

In order to determine whether given model terms should be included or discluded from the model, it needs to test the individual regression co-efficient. Figure 3 A depicts the main effects of pH, temperature and inoculum size on the GA activity. Each individual variable increases the individual regression co-efficient. Each individual variable increases the response with increasing their level up to a certain extent and then further increase in variable level gradually decreases the response. Alternatively,

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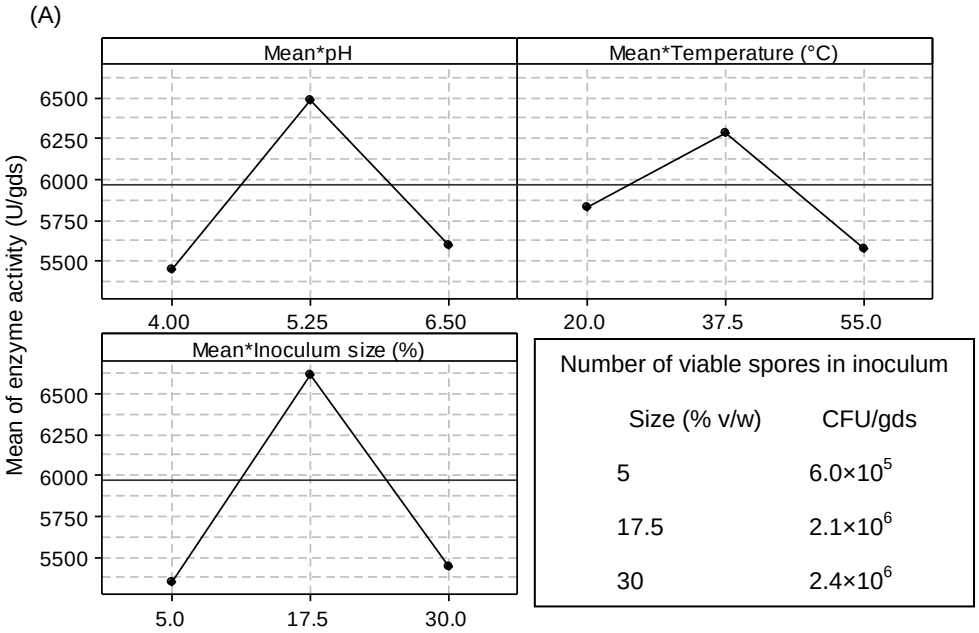
pH and inoculum size individually appear to have a greater effect on the GA activity (U/gds). Consequently, the levels of these variables appear to have a greater effect on the response, hence the levels of each variable's mean values are given in Table 4. Moreover, the quadratic terms and the interaction terms significantly contributed to the response model. For an interaction effect to be significant, at least one of its parent variable should be significant (Wu, 2000). This fundamental principle for factorial effects is called *effect heredity principle*. Since all the three parent main variables are identified as significant, so any of the interaction between the main effects cannot be rule out. Interaction plots are shown in Figure 3 B and each pair of variables provided the following results:



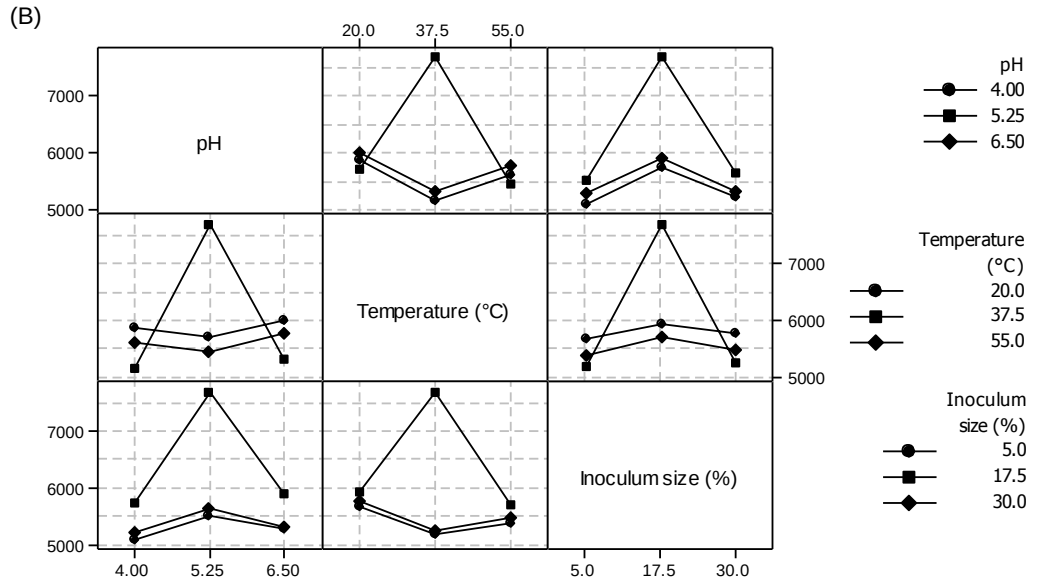


Figure 3: Main (A) and interaction (B) effect plots (Data mean) for GA production (U/gds) from *A. oryzae* IIB-6 in SSF.

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Table 4: Mean values of each level of variables.

Mean values of X1 (pH)

Level	N	Mean	St. Dev.
4.00	4	5439.5	364.0
5.25	7	6488.4	1147.9
6.50	4	5592.0	352.8

Individual 95% CIs For Mean Based on Pooled St. Dev.

```
------+---------+---------+---------+---
(-------------*------------)
(---------*---------)
(------------*------------)
------+---------+---------+---------+---
 4900       5600       6300       7000
```

Enzyme activity (U/gds)

Pooled St. Dev. = 850.4

Mean values of X2 (Temperature, °C)

Level	N	Mean	St. Dev.
20.0	4	5820.5	140.7
37.5	7	6285.3	1333.6
55.0	4	5566.5	172.9

Individual 95% CIs For Mean Based on Pooled St. Dev.

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-----+---------+---------+---------+----
(--------------*--------------)
(----------*----------)
(--------------*-------------)
-----+---------+---------+---------+----
 4900       5600       6300       7000
```

Enzyme activity (U/gds)

Pooled St. Dev. = 949.5

Mean values of X3 (Inoculum size, % v/w)

Level	N	Mean	St. Dev.
5.0	4	5348.8	246.9
17.5	7	6625.9	1018.9
30.0	4	5442.3	241.8

Individual 95% CIs For Mean Based on Pooled St. Dev.

```
-----+---------+---------+---------+----
(----------*-----------)
(--------*-------)
(-----------*----------)
-----+---------+---------+---------+----
 4900       5600       6300       7000
```

Enzyme activity (U/gds)

Pooled St. Dev. = 740.9

X1X2: The interaction of pH (X1) at levels of 4, 5.25, 6.4 and temperature (X2) at levels of 20, 37.5, 55 °C showed that at temperature 20 °C, the pH at all levels gave almost same GA production, but when the temperature increases to 37.5 °C, the pH 4 and 6.4 level decreases the enzyme production. However, at the same temperature, the pH 5.25 gave maximum enzyme activity. A further increase in temperature to 55 °C, resulted in same enzyme production at all the three levels of pH as was attained at 20 °C. The same trend was observed by all the three levels of temperature at each level of pH.

X1X3: The cumulative effect of pH (X1) at levels of 4, 5.25, 6.4 and inoculum size at the levels of 5, 17.5, 30%, showed that at pH 4, the inoculum levels 5 and 30% gave low enzyme production as compared to 17.5%. When the pH was increased to 5.25, the enzyme activity was also increased at 5 and 30% levels of inoculum but this increase was not very prominent. However, 17.5% inoculum level increased the enzyme production to a large extent at the same pH. Further increase in pH up to 6.5 decreased the enzyme activity at all inoculum levels. The same trend was observed by all the three levels of pH at each level of inoculum size.

X2X3: The mutual interaction between temperature (X2) at levels of 20, 37.5, 55 °C and inoculum size (X3) at levels of 5, 17.5, 30% showed that at temperature 20 °C, inoculum size at all the three levels gave almost same enzyme activity. When temperature raised to 37.5 °C,

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enzyme activity was decreased at inoculum levels 5 and 30%, but it was increased and became maximum at the same temperature when inoculum at the level of 17.5% was used. Further increase in temperature upto 55 °C, decreases the enzyme activity at all levels of inoculum. On the other hand, when inoculum level was 5%, almost same enzyme production was observed at all the temperature levels. By increasing the inoculum level to 17.5% enzyme activity was also increased and became maximum at 37.5 °C. Further increase in inoculum level upto 30% decreases the enzyme activity at all temperature levels.

Least square multiple regression

The main and interaction plots are helpful in visualizing which variable/s individually or in combination affect the response most. But in order to determine the significance of the variables, an appropriate statistical test "*t*-test" under least square multiple regression was employed to analyze the variability of data (Table 5). The main effects of pH, temperature and inoculum size were very significant (p<0.0001) and all these variables increases the GA production as they have large positive *t*-values. The sign of "*t*" imply the impact of their effects on enzyme activity. The squared or quadratic effects of all the variables were also very significant (p<0.0001) but they have negative impact on enzyme production. The interaction effects of all the variables were non-significant (p>0.05%). Further the interaction effect X2X3 between temperature (X2) and inoculum size (X3) have zero co-efficient value so was excluded from the model.

Table 5: Co-efficient of main and interaction effects of the variables estimated by multiple linear regression.

Independent variables	Co-efficient (β)	Standard error co-efficient	*t*-value	*p*-value
Constant	-18522.5	199.543	-92.824	<0.0001**
X1	7637.0	65.853	115.969	<0.0001**
X2	179.8	3.256	55.239	<0.0001**
X3	314.9	4.027	78.189	<0.0001**
$X1^2$	-720.1	6.001	-120.001	<0.0001**
$X2^2$	-2.5	0.031	-82.088	<0.0001**
$X3^2$	-8.7	0.660	-145.650	<0.0001**
X1X2	0.2	0.412	0.416	0.694*
X1X3	-1.2	0.577	-2.081	0.092*
X2X3	0.0	0.041	0.694	0.529*

*Non-significant at p>0.05%, **Significant at p<0.05%

Graphical representation

The relationship between independent and dependent variables was graphically represented by 2D (Figures 3A-5A) counter and 3D (Figures 3B-5B) response surface plots generated by the model. Different shapes of the contour plots indicated different interactions between the variables, an elliptical contour plot indicated the significant interactions between the variables while a circular contour plot means otherwise (Zhao *et al*., 2012). There are remarkable changes, which occur due to the pH of the substrates. These are mainly the production of acids due to incomplete oxidation of the substrate or uptake of ammonium ions, which cause the pH to fall, while the release of ammonia by deamination increases the pH. Similarly, microbial growth in SSF generates significant amount of metabolic heat. It has been reported that 100-300 kJ of heat per kg of cell mass is generated in a SSF process. Temperature rises rapidly, because there is little water to absorb the heat or in other words mean specific heat capacity of the fermenting mass is much lower than that of water. Therefore, establishment of pH and temperature gradients for the optimal production of enzyme are very important (Manpreet *et al*., 2005). Figure 4A shows the interaction of pH (X1) and temperature (X2) on enzyme yield when inoculum size was fixed at 17.5%. Increase in pH from 4 to 5.2, improve the enzyme production. However, when pH was over 5.2, there was a gradual decline in the response and incubation temperature over 36 °C did not show any obvious effect on enzyme yield. The optimal GA yield was 7715.11 U/gds achieved at pH 5.29 and temperature 36.21 as predicted by point prediction method (Figure 4B). Figure 5A shows the interaction of pH (X1) and inoculum size (X3) on enzyme production when temperature was fixed at 37.5 °C. By increasing the pH from 4 to 5.2, enzyme production was also increased. However, further increase in pH gradually decreased the enzyme activity. Similarly, enzyme yield was increased with the increase of inoculum size from 5 to 17% and but over 17% gradual decrease in enzyme production was observed. The maximum GA production was 7710.35 U/gds achieved at pH 5.29 with inoculum size of 17.7% (Figure 5B). Figure 6(A) depicts the interaction of temperature (X2) and inoculum size (X3) by keeping the pH (X1) at 5.25. The enzyme production was increased by increasing the incubation temperature from 20 to 36 °C which decreased over 36 °C. The inoculum size over 17.5% did not show any improvement in enzyme yield. The optimum enzyme activity was 7713.67 U/gds at temperature 36.22 °C and 17.96% of inoculum size (Figure 6B). Varzakas *et al*. (2008) optimized 2×10^7 conidia/gds and 37 °C for the production of enzyme from *A. niger*. Bhatti *et al*. (2007) reported inoculum size of 15% (w/v) with 10^6-10^7 spores/mL,

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incubation temperature of 35±1 °C and pH 5.0 for the production of enzyme from *Fusarium solani* by using wheat bran. *A. oryzae* HS-3 showed optimum productivity in a temperature range of 30-40 °C on wheat bran (Singh and Soni, 2001).

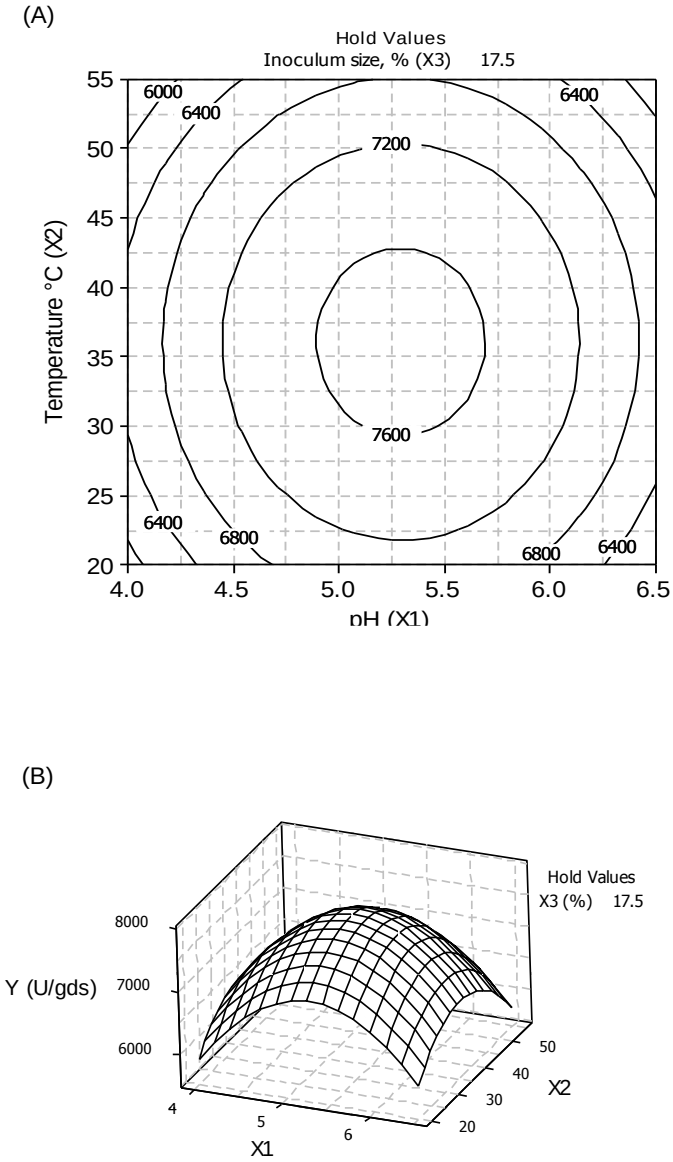

Figure 4: Counter plot (A) and response surface plot (B) for the production of GA (U/gds) from *A. oryzae* IIB-6 showing the interactive effect of pH (X1) and temperature (X2) in SSF by keeping inoculum size (X3) at central level.

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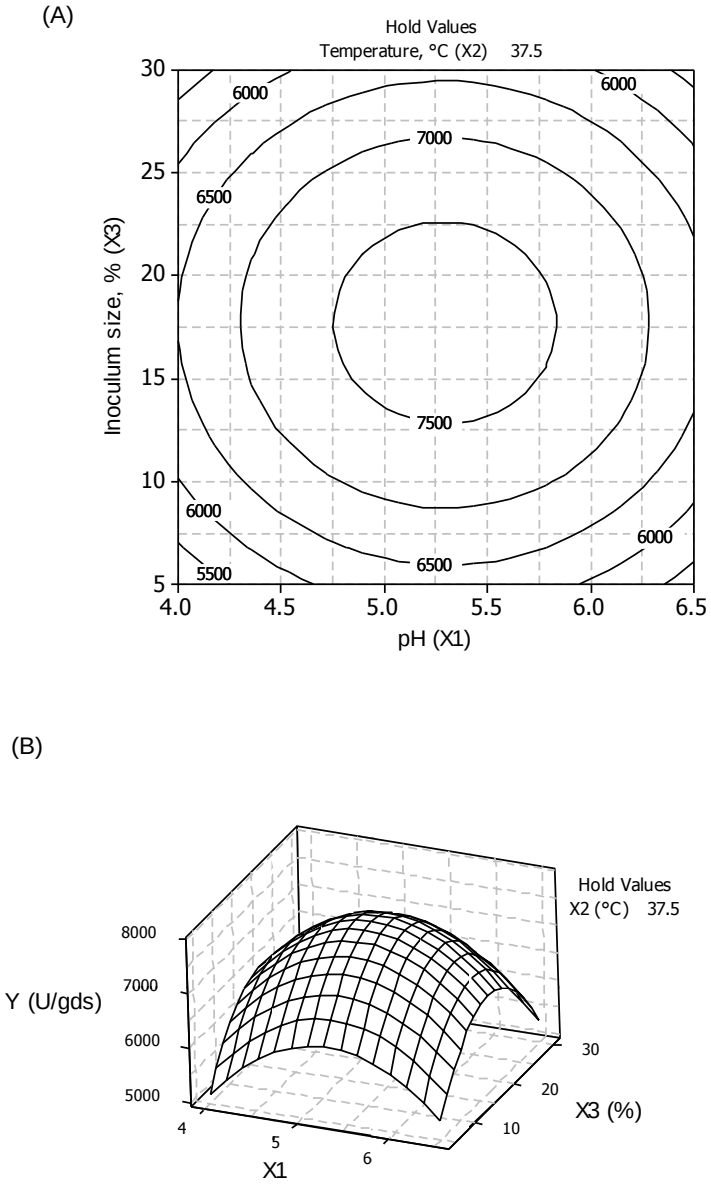

Figure 5: Counter plot (A) and response surface plot (B) for the production of GA (U/gds) from *A. oryzae* IIB-6 showing the interactive effect of pH (X1) and inoculum size (X3) in SSF by keeping temperature (X2) at central level.

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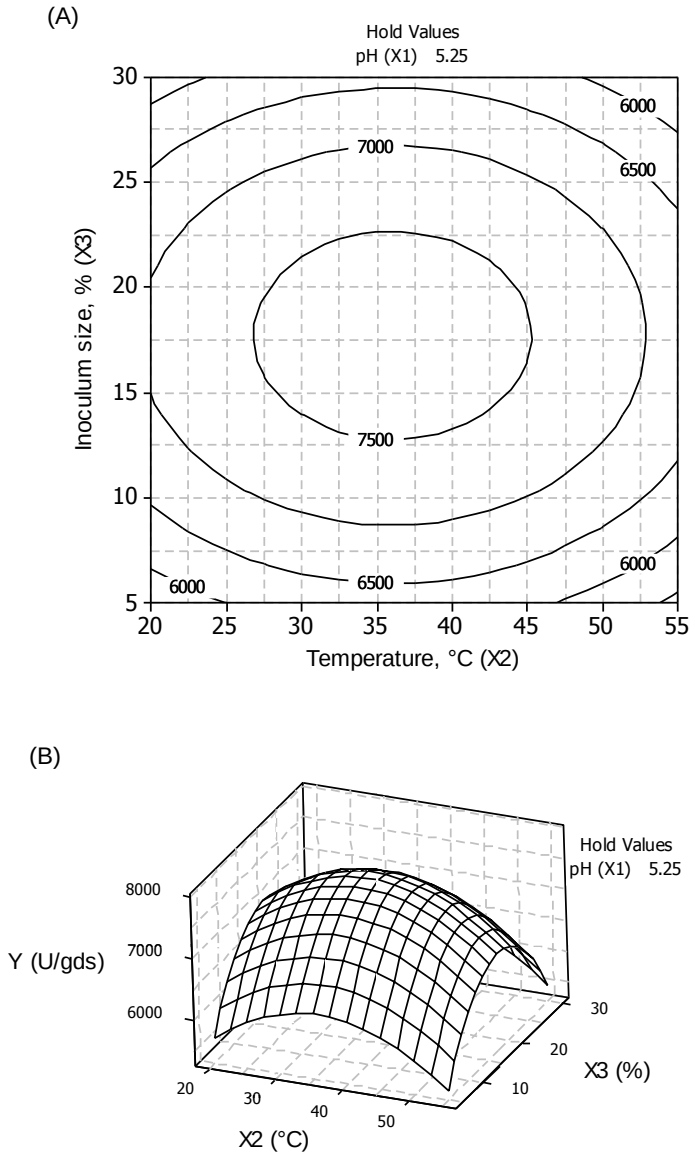

Figure 6: Counter plot (A) and response surface plot (B) for the production of GA (U/gds) from *A. oryzae* IIB-6 showing the interactive effect of temperature (X2) and inoculum size (X3) in SSF by keeping pH (X1) at central level.

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Model validation

With the help of counter plots and point prediction the optimum value of the combination of the three fermentation variables was determined for maximum production of enzyme. In order to verify the optimization results and to validate the developed second order quadratic model, an experiment was conducted under predicted conditions (Table 6). The closeness of the predicted response for GA (7713.04 U/gds) and the experimental response (7750.02 U/gds) proved the validity of the model. This indicated that the optimization achieved in the present study was reliable.

Table 6: Predicted and experimental value of responses at optimum conditions.

Optimum conditions	pH	Temperature (°C)	Inoculum size (%)	GA activity (U/gds)
Predicted	5.29	36.22	17.70	7713.04
Actual	5.30	37.00	17.00	7750.02

Standard error (S.E) = 18.5

CONCLUSION

Maltose and peptone were found as the best additional carbon and nitrogen supplements of wheat bran for GA production. Application of RSM for optimization studies is an effective method for improving the enzyme production and also understanding the interaction effects between the variables with less number of experiments. Box-Behnken factorial design was successfully employed to optimize physical parameters of SSF. Under optimal conditions, GA activity of 7750.02 U/gds was obtained and it is closer to the predicted value. Additionally, an R^2 of 0.9999 indicates that the regression equation explains 99.99% of the variance in the dependent variables.

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